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In Situ Transfer of Antibiotic Resistance Genes from Transgenic (Transplastomic) Tobacco Plants to Bacteria

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Interkingdom gene transfer is limited by a combination of physical, biological, and genetic barriers. The results of greenhouse experiments involving transplastomic plants (genetically engineered chloroplast genomes) cocolonized by pathogenic and opportunistic soil bacteria demonstrated that these barriers could be eliminated. The *Acinetobacter* sp. strain BD413, which is outfitted with homologous sequences to chloroplastic genes, coinfected a transplastomic tobacco plant with *Ralstonia solanacearum* and was transformed by the plant's transgene (*aadA*) containing resistance to spectinomycin and streptomycin. However, no transformants were observed when the homologous sequences were omitted from the *Acinetobacter* sp. strain. Detectable gene transfer from these transgenic plants to bacteria were dependent on gene copy number, bacterial competence, and the presence of homologous sequences. Our data suggest that by selecting plant transgene sequences that are nonhomologous to bacterial sequences, plant biotechnologists could restore the genetic barrier to transgene transfer to bacteria.

The tremendous adaptation potential of prokaryotes is mainly related to their ability to exchange genes by specific mechanisms such as conjugation, transduction, and transformation (22). The efficiency of such mechanisms during bacterial incorporation of genes from transgenic plants, and particularly those encoding antibiotic resistance, is difficult to assess (15), although the occurrence of such transfers under natural soil conditions would remain rare (3). In soil, in spite of the persistence of plant DNA, there would be relatively few naturally transformable bacteria (13, 17) and these prokaryotes would rarely find the required conditions to develop competence (4), thus apparently significantly reducing the probability of gene transfer. For those bacteria that have developed specific symbiotic or pathogenic relationships with plants, conditions for gene transfer could be favorable, as shown with the plant pathogen *Ralstonia solanacearum* (2). This bacterium multiplied in its host plant, disorganized tissues, and colonized the plant via the vascular tissue, leading to the development of a competence stage of active transformability in planta (2). Although bacteria-bacteria gene transfer occurred between *R. solanacearum* in planta, no gene transfer from the transgenic plant to the *R. solanacearum* was detected. This lack of detection was not necessarily due to the lack of transfer but was possibly due in part to the low transformation efficiency of *R. solanacearum* and the dilution of the transgene by the entire plant genome (3). The ratio between target versus non-target DNA sequences on which homologous recombination can occur was very low, thereby possibly preventing the integration mechanism necessary to produce a measurable number of transformants.

While the potential of plant environments to mediate plant-bacteria gene exchange has not been established, two recent events have increased the likelihood. Genetic engineering of the chloroplast genome led to a new generation of transgenic plants (21). These transplastomic plants were designed to prevent dissemination of the transgene via pollen and to increase the number of gene copies in the transgenic plant (8). Transplastomic plants harbor around 10,000 copies of the transgene per cell (1, 7), which can be compared to less than 10 for traditional and nuclear-modified transgenic plants.

The second event was the discovery that plants can also be colonized by soil bacteria which exhibit much higher transformation frequencies than *R. solanacearum*. This is the case for the opportunistic soil bacterium *Acinetobacter* sp. strain BD413, which was found to actively colonize *R. solanacearum*-infected plants and therein develop a competence state (12). Thus, given the development of transplastomic plants, we focused our efforts on determining whether *Acinetobacter* sp. could incorporate DNA sequences from plant transgenes in planta. In order to optimize the possible gene transfer, the *Acinetobacter* sp. used was outfitted with a plasmid containing homologous sequences to the chloroplast genome. Clearly, our model used plant sequences in the recipient bacteria, whereas under other natural conditions the transgene's prokaryote origin would provide it with homologous sequences to indigenous bacteria. While conceptually similar, our model enabled a more sensitive isolation of the transformants.

MATERIALS AND METHODS

Strains, culture conditions, and plasmids. *Acinetobacter* sp. strain BD413 (DSM586) was cultured on Luria Bertani medium (10 g of Bacto tryptone, 5 g of yeast extract, and 5 g of NaCl in 1 liter of distilled water) containing 20 μg of nalidixic acid (Sigma Chemical Co., St Louis, Mo.) ml^{-1}. *R. solanacearum* K60 was cultured in rich BG medium (5) supplemented with 12 μg of gentamicin (Sigma) ml^{-1}. Both strains were cultured in liquid and on solid media at 28°C for 48 h. Plasmid pLEP01 (D. Rumeau and G. Peltier, personal communication) was an ampicillin-resistant cloning vector which contained the *aadA* gene, conferring resistance to spectinomycin and streptomycin, flanked with plastid sequences corresponding to the *rbcL* and *accD* regions. Plasmid pFB3, which contained an

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nptII gene deleted from the plasmid pBin19 (3), was introduced into *Acinetobacter* sp. strain BD413 by natural transformation.

Construction of transplastomic and nuclear plants. Plastid transformants were generated by particle bombardment of *Nicotiana tabacum* cv. PBD6 essentially as described by Staub and Maliga (21). Green calli were selected on growth media containing 500 mg of spectinomycin dihydrochloride per liter until they formed shoots. The shoots were rooted on MS medium (21) also containing spectinomycin as described above. In order to confirm integration of *aadA* in the plastid genome, PCR analyses were conducted with spectinomycin-resistant plant DNA by using one primer complementary to part of the *rbcL* gene immediately outside the homologous recombination region while another one targeted the chimeric plastid promoter of the *aadA* cassette. For nuclear plants, we used the tobacco pKHG3 developed by Bertolla et al. (3).

Construction of the pBAB2 plasmid. A standard PCR protocol was used to amplify 2,574 bp by using DNA extracted from a wild tobacco plant as a template and the primers chloro1 (5′-GGTAGAGCCGTTTATGAA-3′) and chloro2 (5′-GCTCCTCCTCCGAATGAA-3′), which are complementary to part of the plastid *rbcL* and *accD* regions, respectively. The amplified region extends from position 58150 to position 60724 of GenBank accession number Z00044. The PCR products were cloned in a pUC18 cloning vector providing plasmid pBAB1, which was subsequently digested by *Eco*RI and *Hin*dIII restriction enzymes. The cohesive ends of the insert allowed an efficient cloning into electroporated DH10B *Escherichia coli* cells by using an adequately restricted RSF1010-derived broad-host-range vector (pMMB190) (14). The presence of the expected recombinant pBAB2 plasmid in the growing *E. coli* colonies was confirmed by standard restriction and PCR techniques, and this plasmid was subsequently introduced into *Acinetobacter* sp. strain BD413 by natural transformation.

Transforming DNA. Plasmid and plant DNAs were purified with the appropriate kits (Qiagen, Mannhein, Germany) according to recommendations from the manufacturer. Fresh plant material was also used for in vitro transformation assays. Two grams (wet weight) of either the central vein from the leaf or the foliar parenchyma was crushed in 10 ml of distilled sterile water with an Ultra-Turrax T25 homogenizer operating at 25,000 rpm (IKA-Werke GmbH and Co., Staufen, Germany). Aliquots of purified DNA and fresh plant material were used for in vitro transformation experiments.

In vitro transformation experiments. *Acinetobacter* sp. strain BD413(pBAB2) was cultured overnight with agitation before an aliquot was diluted 25-fold into fresh liquid medium and cultured for an additional 2 h to reach the competent state as described by Palmen et al. (18). A final amount of 1 μg of plasmid pLEP01 or 5 μg of purified plant DNA in a volume of 40 μl was used to transform 360 μl of competent *Acinetobacter* sp. strain BD413(pBAB2) cells. In other cases, 300-μl aliquots of crushed plant suspension (central vein or foliar parenchyma) were added to 2,700 μl of competent cells. Mixtures were incubated for an additional 90 min at 28°C for DNA uptake before plating on Luria Bertani medium supplemented with the appropriate antibiotics.

Bacterial inoculation in planta. Ten milliliters of each overnight bacterial culture was harvested twice, and then the cells were resuspended in sterile water to obtain two stock solutions with an optical density at 600 nm of 1 for *R. solanacearum* and an optical density at 600 nm of 1.4 for *Acinetobacter* sp. strain BD413(pBAB2). The final inoculum was prepared by mixing 1 ml of the *R. solanacearum* stock suspension with 2 ml of the *Acinetobacter* sp. stock suspension in a final volume of 20 ml of sterile distilled water. Two milliliters of this inoculum was injected into the central vein of the tobacco leaf. Plant infections by bacterial strains were performed on 1-m-high plants, with the central stems of the leaves being approximately 30 cm long. Infected plants were incubated in a growth chamber at 28 ± 2°C with a light regimen consisting of 16 h of light and 8 h of darkness at 70% relative humidity.

Infection kinetics of bacterial strains. The infected stems were removed at different times after inoculation to be crushed in sterile distilled water. The protocol to extract bacterial cells from plant tissues was identical to that used previously by Kay et al. (12) except for the DNase treatments. The infected stems were removed to be crushed and homogenized in sterile distilled water with an Ultra-Turrax T25 homogenizer operating at 25,000 rpm. Eight milligrams of DNase I (Roche Diagnostics GmbH, Mannhein, Germany) was added per gram of fresh plant tissue, and this suspension was then incubated at 37°C for 20 min before being plated on Luria Bertani and BG medium. Then, 100 μl of the appropriate dilution of plant tissue suspensions was plated on Luria Bertani medium with the appropriate antibiotics in order to enumerate the *R. solanacearum* and *Acinetobacter* sp. strain BD413(pBAB2) cells. Putative transformant cells of *Acinetobacter* sp. strain BD413(pBAB2) were detected by plating the remaining plant tissue suspensions on Luria Bertani medium supplemented with nalidixic acid (20 μg ml^{-1}), ampicillin (50 μg ml^{-1}), and spectinomycin (100 μg ml^{-1}). In order to limit fungal contamination, the media were supplemented with cycloheximide (200 μg ml^{-1}).

Clone characterization. PCR amplifications were performed directly on colonies picked up from selective media plates and resuspended in 20 μl of sterile distilled water. Experimental conditions consisted of transferring 29 μl of sterile water, 5 μl of 10× PCR buffer (Gibco-BRL/Life Technologies, Inc., Gaithersburg, Md.), 2.5 μl of 1% detergent W-1 solution (Gibco-BRL), 1 μl of 50 mM magnesium chloride, 5 μl of 10 mM deoxynucleoside triphosphate mix (Pharmacia), and 2.5 μl (each) of 10 μM primers into a PCR tube. Then, 0.25 μl of *Taq* DNA polymerase (Gibco-BRL) and 1 μl of cell suspension were added. Amplifications were carried out in a Perkin-Elmer 7200 thermocycler for 35 cycles, each of which consisted of a denaturation step at 94°C for 45 s, an annealing step at 55°C for 45 s, and an elongation step at 72°C for 1 min.

PCR was conducted with primers complementary to part of the 16S ribosomal DNA gene (pA, 5′-AGAGTTTGATCCTGGCTCAG-3′, and pH, 5′-AAGGAGGTGATCCAGCGCCA-3′) (10). The 1,600-bp fragment obtained was then digested with the *Hae*III restriction enzyme (Gibco-BRL), and the resulting patterns were compared to those of the recipient strain.

PCR was conducted with primers FGPaad1172 (5′-ATTCCGTGGCGTTAT-3′) and FGPaad1554 (5′-TGACGGGCTGATACT-3′) (2), which are complementary to part of the *aadA* gene, in order to monitor the presence of the marker gene in the transformant clones. Other PCRs were performed on the chimeric region (plastid and marker genes) with a combination of the primer FGPaad1554 and chloro1. This latter primer is complementary to part of the *rbcL* gene (plastid region used for homologous recombination in pBAB2). The 1,900-bp fragment obtained was digested with the restriction enzyme *Rsa*I, and then restriction patterns were compared to that obtained with purified plant DNA amplification.

Hybridization protocol. Dot blotting hybridizations were performed with oligoprobe FGPaad1554 on recombinant plasmids extracted from transformant clones. Plasmid pLEP01 (30 ng) and plant DNA (250 ng) were used as positive controls, and pBAB2 (30 ng) was used as a negative control. All DNA was previously denatured before being applied to a nylon membrane (GeneScreen Plus; NEN, Boston, Mass.) in a Bio-Rad apparatus. Prehybridization and hybridization were performed at 41°C for 14 h with [γ-^{32}P]dATP-labeled oligoprobe according to the method of Simonet et al. (20). Membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature, once in 2× SSC plus 0.1% sodium dodecyl sulfate buffer for 30 min at 41°C, and then in 1× SSC plus 0.1% sodium dodecyl sulfate buffer for 30 min.

Control experiments. A first set of control experiments involved determining whether cells of *Acinetobacter* inoculated into the plant were in a competence state at the moment of injection. An aliquot of bacterial inoculum (900 μl) was mixed with 2 μg of plasmid pLEP01 and treated for natural transformation according to the standard protocol (18) previously used by Kay et al. (12). DNase I was added to the mixture after incubation at 28°C for 90 min, just before plating on selective media.

A second set of control experiments was conducted to assess the capacity of DNase I to degrade the totality of plant DNA released in the crushed plant suspension, as this could potentially transform *Acinetobacter* sp. ex planta. An aliquot of 900 μl of competent cells was added to crushed plant suspension (10 ml) with 5 μg of pLEP01 with or without DNase I (8 mg g of fresh $tissue^{-1}$). Then the mixtures were incubated for 20 min at 37°C before being plated on selective media to detect the presence or absence of transformant, depending on the presence or absence of DNase I.

RESULTS AND DISCUSSION

The occurrence of several successive biological events is required before a gene from transgenic plants can be expressed in a bacterium: (i) release of genes during plant cell degradation, (ii) presence of transformable bacteria, (iii) incorporation of the transgene in bacterial DNA, and (iv) expression of the gene in the bacteria. The model system presented here was optimized such that each step either separately or as a continuous chain of events lacked some barriers to gene transfer. The first series of experiments examined these steps individually (see "Stepwise demonstration" below) and the last experiment allowed all of these steps to occur together in order to examine

TABLE 1. Influence of DNA source and transformation conditions on efficiency of natural transformation of *Acinetobacter* sp. strain BD413 by transgenic plant DNA

Recipient strain	In vitro transformation frequency of[a]:				In planta transformation of[b]:	
	Plasmid pLEP01[e]	Pure plant DNA	Crushed plant tissues (vein)	Crushed plant tissues (foliar)	Nuclear transgenic plant[f]	Plastid transgenic plant
Acinetobacter sp. strain BD413[c]	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	$<10^{-8}$	0	0
Acinetobacter sp. strain BD413(pBAB2)[d]	$(6 \pm 3.4) \times 10^{-3}$	$(4.1 \pm 2.3) \times 10^{-6}$	$(2.6 \pm 2.1) \times 10^{-8}$	$(6.4 \pm 2.8) \times 10^{-8}$	0	31

[a] Number of transformants per recipient cell ± standard deviation.
[b] In planta transformation is expressed as the total number of transformants. For the combination transplastomic plants, *Acinetobacter* sp. strain BD413(pBAB2), experiments were conducted five times and independently. Three of the five plants produced transformants. Out of the 21 independent central leaf veins harvested from the three plants, 8 produced 31 transformants.
[c] *Acinetobacter* sp. strain BD413 without any sequence to promote homologous recombination with plant DNA.
[d] *Acinetobacter* sp. strain BD413(pBAB2) harboring plastid-borne sequences on which homologous recombination could occur.
[e] Plasmid pLEP01 cannot replicate in *Acinetobacter* sp. strain BD413. Transformants resulted from homologous recombination-based integration events only.
[f] Based on the use of nuclear transgenic tomato plants, pKHG3, and *Acinetobacter* sp. strain BD413 harboring or not harboring plasmid pFG4ΔnptII (16), homologous recombination could potentially occur on *nptII* sequences (see reference 3 for details).

the probability of the chain of events occurring in planta (see "Complete gene transfer" below).

Stepwise demonstration. First, we determined whether the *aadA* gene flanked by some of the plant chloroplast genome could act as donor DNA for the transformation of a specifically constructed recipient bacterium containing these plant chloroplast sequences. This system was useful for selecting transformants and simulated what could occur with any other transgene sequence exhibiting similarity with bacterial genes. We constructed and transferred an RSF1010 (14) derivative plasmid (pBAB2) containing tobacco plastid sequences, including the genes *rbcL* and *accD*, to *Acinetobacter* sp. and tested for homologous recombination-mediated integration of the *aadA* gene when transformed with plant DNA. In vitro transformation tests of competent *Acinetobacter* sp. harboring pBAB2 were performed with the plasmid pLEP01, which cannot replicate in *Acinetobacter* sp. and was used to transform the plants. This plasmid is similar to pZS19 (21). Transformants resulting from integration events were detected at frequencies up to 6×10^{-3} (Table 1), confirming the efficiency of the recipient plasmid-based sequences to process recombination. The following step was to use total DNA extracted from transplastomic plants to transform the *Acinetobacter* sp. harboring pBAB2. Compared to the previous results, transformation frequencies dropped to 4.1×10^{-6} (Table 1), confirming a limiting effect due to the dilution of the target sequences among the large plant genome. However, frequencies were higher than those observed for the same recipient strain by Gebhard and Smalla (11) when they conducted transformation tests with DNA extracted from nuclear-modified plants. The higher transgene copy number in transplastomic plants relative to conventional transgenic plants probably increased the probability of these sequences to be transferred to bacteria by natural transformation. Moreover, these results confirm the interest in using the soil bacterium *Acinetobacter* sp. strain BD413 as a recipient since it exhibits higher transformation frequencies than *R. solanacearum*, which failed to produce any detectable transformants when exposed to DNA extracted from nuclear-modified plants (3).

Next, experiments were conducted to demonstrate the availability and transformability of DNA released by the plant cells. First, we crushed plant tissue (both the central vein and the foliar parenchyma) in order to use transplastomic plant DNA for transforming the recipient *Acinetobacter* sp. strain BD413, which contained the pBAB2 plasmid. Positive detection of transformants confirmed previous results from Gebhard and Smalla (11) about the potential of nonpurified plant DNA to act as donor DNA, suggesting that neither histones, other plant proteins, nor the specific conformation of plant DNA inhibited bacterial transformation (Table 1). Moreover, previous results (3) indicated that a plant infected by *R. solanacearum* developed wilting symptoms and released DNA close to the infecting bacteria, confirming that transgene sequences become accessible to bacteria.

Finally, the in situ transfer of plant genes to microorganisms requires not only the presence of the recipient bacteria in contact with the donor DNA but also the environmental conditions in which such bacteria can develop a physiologically active competence state. Previous results indicate that *Acinetobacter* sp. acts as an opportunistic organism cocolonizing plants infected by the *R. solanacearum* pathogen (12). In addition, this active colonization, which led to *Acinetobacter* sp. population levels of up to 10^6 cells g of fresh plant tissue^{-1}, was concomitant with the development of a competence state. Tests were performed in order to ensure that the experimental conditions used could only lead to transformation in planta. Such controls required the demonstration that *Acinetobacter* sp. cells in the inoculum were not yet competent and that this physiological state developed during plant colonization. This was done by mixing *Acinetobacter* sp. cells ready for cocolonization with plasmid pLEP01 DNA in vitro and then submitting this suspension to the transformation protocol, effectively avoiding the in planta step. We did not detect any transformant in these controls, whereas the same number of *Acinetobacter* sp. cells grown to be transformable exhibited the usual measurable transformation rate. According to previous data (12), *Acinetobacter* sp. competence is reached after 4 days of cocolonization and is, thus, fortuitously concomitant to the release of DNA in decaying plant tissues.

Controls were also necessary to demonstrate that transfor-

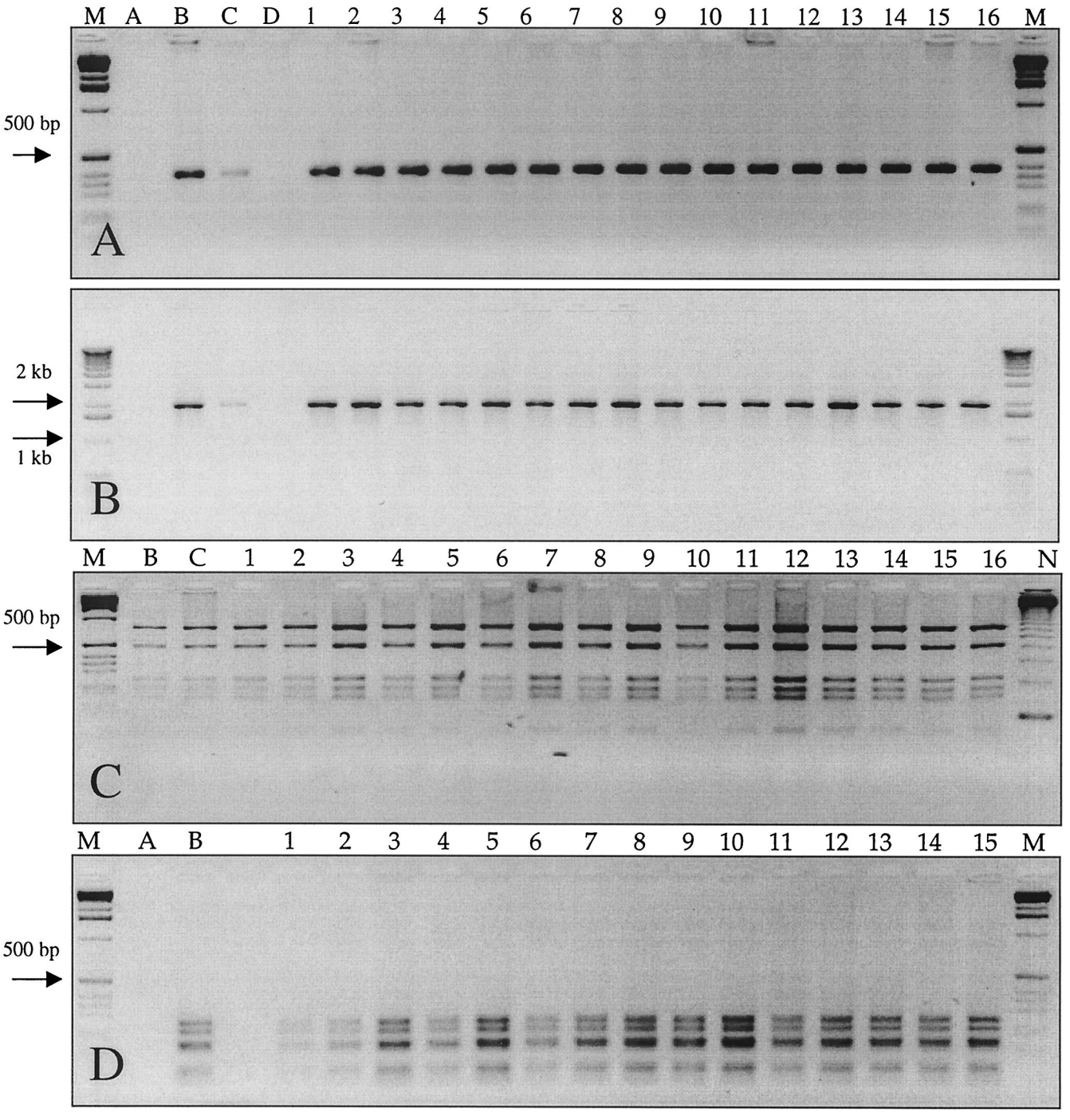

FIG. 1. (A and A′) PCR-based control of the presence of the plastid *aadA* marker gene in the genome of *Acinetobacter* sp. strain BD413(pBAB2) transformants. Primers FGPaad1172 and FGPaad1554 (2) are complementary to part of the *aadA* gene and amplify a 382-bp-long DNA fragment. Lanes M contained 1-kb size marker ladders. Lanes A to D, controls with the templates sterile H_2 O (A), pLEP01plasmid DNA (B), plastid plant DNA (C), and recipient *Acinetobacter* sp. strain BD413(pBAB2) DNA (D). Lanes 1 to 31, PCR products resulting from amplifications with DNA from *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in planta. Lane T, PCR products resulting from amplifications with DNA from one of the numerous *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in vitro. (B and B′) PCR-based control of the presence of the plastid-specific chimeric construction in the genome from *Acinetobacter* sp. strain BD413(pBAB2) transformants. Primer chloro1, which is complementary to part of the *rbcL* gene (a site for homologous recombination), was used in combination with FGPaad1554 (2), which is complementary to the part of the *aadA* gene, to amplify a 1.9-kbp-long DNA fragment. Lanes M contained 1-kb size marker ladders. Lanes A to D, controls with the templates sterile H_2 O (A), pLEP01 plasmid DNA (B), plastid plant DNA (C), and recipient *Acinetobacter* sp. strain BD413(pBAB2) DNA (D). Lanes 1 to 31, PCR products resulting from amplifications with DNA from *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in planta. Lane T, PCR products resulting from amplifications with DNA from one of the numerous *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in vitro. (C and C′) Restriction fragment length polymorphism (RFLP) (enzyme *Rsa*I)

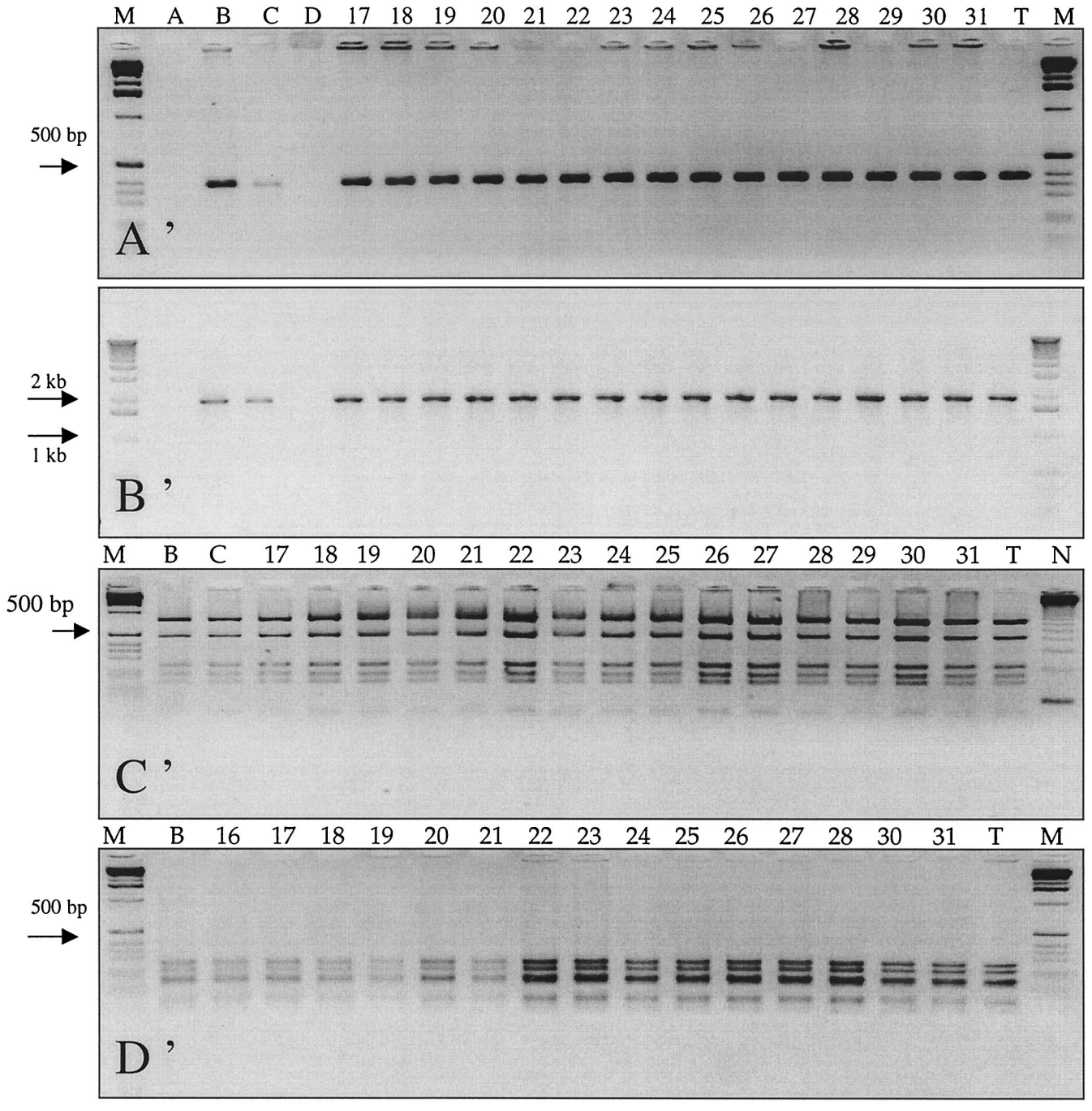

FIG. 1—*Continued*.

of PCR products resulting from positive amplifications with the primers chloro1 and FGPaad1554, which target the plastid chimeric construction. Lanes M contained 1-kb size marker ladders. Lanes B and C, controls with templates pLEP01 plasmid DNA (B) and plastid plant DNA (C). Lanes 1 to 31, PCR products resulting from amplifications with DNA from *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in planta. Lane T, PCR products resulting from amplifications with DNA from one of the numerous *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in vitro. Lane N is a marker ladder containing a 123-bp marker. (D and D′) RFLP (restriction enzyme *Hae*III) of PCR products resulting of amplifications with primers pA (5′-AGAGTTTGATCCTG GCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCGCCA-3′), which target the ribosomal genes. Lanes M contained 1-kb size marker ladders. Lanes A and B, controls with the templates sterile H_2O (A) and recipient *Acinetobacter* sp. strain BD413(pBAB2) DNA (B). Lanes 1 to 31, PCR products resulting from amplifications with DNA from *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in planta. Lane T, PCR products resulting from amplifications with DNA from one of the numerous *Acinetobacter* sp. strain BD413(pBAB2) spectinomycin-resistant clones after a gene transfer occurred in vitro. The similarity of the patterns between the recipient strain and the spectinomycin-resistant clones indicate that they are all the same bacterium, *Acinetobacter* sp. strain BD413.

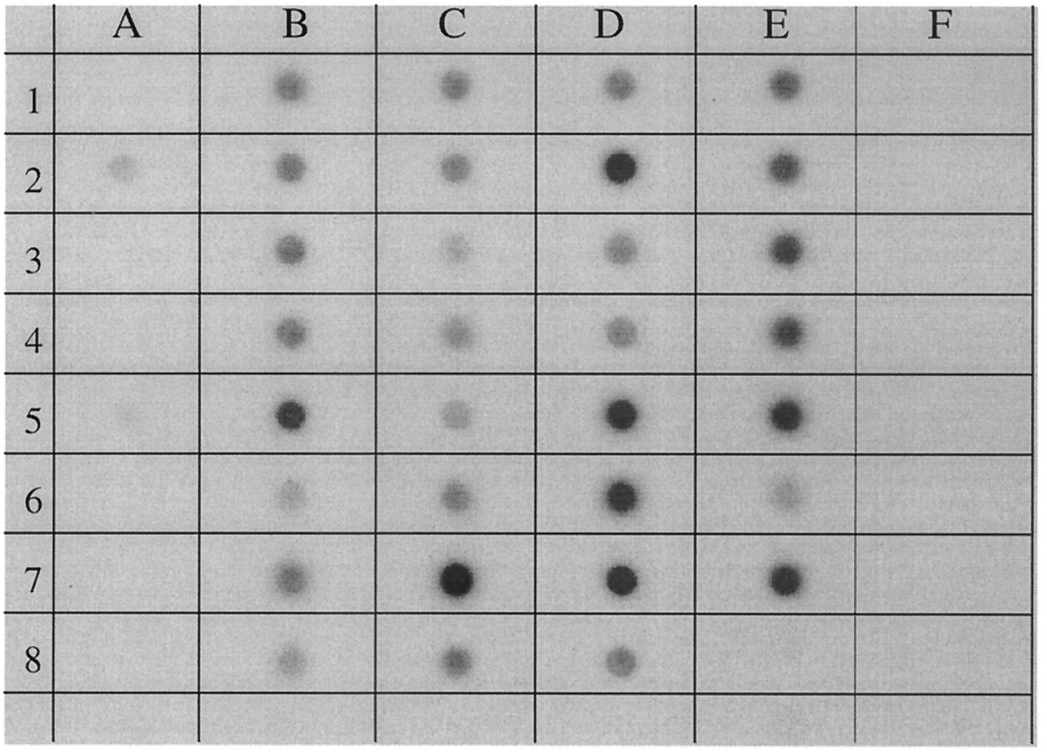

FIG. 2. Hybridization-based control of the presence of the plastid *aadA* marker gene in the genome of *Acinetobacter* sp. strain BD413(pBAB2) transformants. The oligoprobe consisted of the oligonucleotide FGPaad1554, which is complementary to part of the *aadA* gene. Dot blot hybridizations (standard protocol, hybridization temperature of 41°C) were conducted on plasmids extracted from transformants (Qiagen extraction protocol). Clone A2, positive-control plasmid pLEP01 (30 ng); clone A5, positive-control plastid plant DNA (250 ng); clone F8, negative-control plasmid pBAB2 (30 ng). Clones B1 to B8, C1 to C8, D1 to D8, and E1 to E7 correspond to hybridization on plasmid DNA from transformants of *Acinetobacter* sp.

mation could not occur after the crushing of the plant or during the transformant counting. Since crushed plant suspensions contain nucleic acids released by the plant, this DNA might transform *Acinetobacter* sp. cells under conditions of active growth on culture media. Protocols included a DNase I treatment of the crushed plant suspension in order to degrade any remaining DNA, thus preventing false positives to be produced ex planta. Experiments were performed to test the efficiency of this treatment. Plants were infected with *R. solanacearum* K60 for 12 days. During the crushing step, the plant suspensions were submitted to all combinations of treatments including with or without inoculation of 10^7 competent *Acinetobacter* sp. cells, a 2-μl addition of 5 μg of the plasmid pLEP01, and a 3-μl addition of DNase I. For conditions in which competent *Acinetobacter* sp. strains were mixed with the plasmid pLEP01, DNA transformants were detected at frequencies of up to 1.7×10^{-5} (400 transformants ml^{-1}). However, their numbers dropped below the detection limit when DNase I was added to the plant suspension. In all controls where the DNase I treatment was applied, no transformants were detected. When the plant suspension was inoculated with competence-induced *Acinetobacter* sp. in the absence of DNase I treatment, transformants were detected, confirming the availability of naturally released plant DNA for bacterial transformation.

Complete gene transfer. The data presented above confirm the interest of the biological model based on transplastomic tobacco plants infected by *R. solanacearum* and cocolonized by *Acinetobacter* sp. when investigating gene transfer between transgenic plants and bacteria. The combination of the transformation properties of *Acinetobacter* sp., with its plasmid-borne homologous sequences, and the high chloroplast-based transgene-to-plant genome DNA ratio led to a dramatic increase in bacterial transformation frequencies by plant DNA (Table 1). Moreover, there is interest in such a model not only because of its use under well-controlled laboratory conditions but also because it simulates plants growing in their natural environment where the accumulation of successive events within the plant are required in order for gene transfer to occur. Previous results (12) demonstrated that both a soil pathogen such as *R. solanacearum* and other bacteria, including *Acinetobacter* sp., can infect and colonize plants via their roots. Therefore, all the elements for gene transfer occurring in planta have been individually verified, and their frequencies are sufficiently high to possibly detect the event under natural conditions.

In order to attempt to detect transformants for the whole uninterrupted process, several transplastomic tobacco plants were infected with the *R. solanacearum* and *Acinetobacter* sp. strain BD413(pBAB2) inoculum in the course of five indepen-

dent and time-separated experiments. Plants were incubated until they were wilting before being crushed, being treated with DNase I, and having the suspension plated on selective media. Our investigations focused on the five plants that exhibited wilting symptoms. Thirty-two central leaf veins were harvested, crushed, and treated independently. Eight (25%) of these veins had detectable *Acinetobacter* sp. clones, which exhibited the spectinomycin resistance phenotype expected in the case of a successful gene transfer from the plant to the bacterium. A total of 31 clones were isolated from the eight positive veins. The eight positive veins came from three of the five plants.

Plasmid restriction patterns (results not shown), PCR targeting the *aadA* gene (Fig. 1A and A′) or the chimeric construction (Fig. 1B, B′, C, and C′), and hybridization (Fig. 2) tests confirmed that the *aadA* sequences missing in the recipient strain had been acquired by the spectinomycin-resistant clones and were localized on plasmid pBAB2 inside the plastid sequences, as expected for a homologous recombination-based integration event. Moreover, amplifying the ribosomal sequences of these clones and comparing the *Hae*III restriction patterns of these products to those obtained with the recipient strain confirmed that the 31 isolated clones were *Acinetobacter* sp. strains (Fig. 1D and D′).

The experimental conditions used did not differentiate between isolated bacteria resulting from independent transfer events or from a clonal multiplication of rare transformants. In addition, in planta conditions were too heterogeneous to calculate transfer frequencies precisely. Nevertheless, in the case of clonal multiplication, the minimum number of transformants would be eight, based on the discovery of transformants in eight different veins. Thus, transformation frequencies were probably higher than 10^{-8} for those plants which led to the isolation of transformants. This is higher than that expected to occur with the other biological models, such as *Erwinia chrysanthemi* (19), *Agrobacterium tumefaciens* (6), and *R. solanacearum* (3) in plant environments as well as other *Acinetobacter* sp. strains (16) and *Pseudomonas stutzeri* (9) directly in soil. Our results confirm that gene transfer between transgenic plants and bacteria could occur under natural conditions at least when certain natural barriers are nonexistent. Thus, the greater gene number available with transplastomic plants and the sequence homology used in our experiments increased the frequency of transformation to a detectable number while other combinations, including the use of recipient bacterial strains lacking homologous sequences or nuclear transgenic plants, did not produce detectable transformants (Table 1). In our experiments, the plant-bacteria homologous sequence combination was specifically designed to improve transformant detection. However, our model of what could happen naturally for any transgene exhibiting sequence similarity with bacterial genomes simulates all the transgenes containing prokaryotic genes, such as antibiotic resistance genes.

Evaluations of the likelihood of gene transfer under less optimum conditions and of the likelihood that these optimum conditions could occur (transplastomic plants, highly transformable bacteria, homologous sequences) are the next steps toward estimating the impact of transgenic plants on the soil microbial ecology.

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